

# Tonic neuromodulation of the inspiratory rhythm generator

**Fernando Peña-Ortega\***

*Departamento de Neurobiología del Desarrollo y Neurofisiología, Instituto de Neurobiología, UNAM-Campus Juriquilla, Querétaro, Mexico*

## Edited by:

Francisco Fernandez De-Miguel,  
Universidad Nacional Autónoma de  
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## \*Correspondence:

Fernando Peña-Ortega,  
Departamento de Neurobiología del  
Desarrollo y Neurofisiología, Instituto  
de Neurobiología, Universidad  
Nacional Autónoma de México,  
Boulevard Juriquilla 3001, Querétaro  
76230, México.  
e-mail: jfpena@unam.mx

The generation of neural network dynamics relies on the interactions between the intrinsic and synaptic properties of their neural components. Moreover, neuromodulators allow networks to change these properties and adjust their activity to specific challenges. Endogenous continuous (“tonic”) neuromodulation can regulate and sometimes be indispensable for networks to produce basal activity. This seems to be the case for the inspiratory rhythm generator located in the pre-Bötzinger complex (preBötC). This neural network is necessary and sufficient for generating inspiratory rhythms. The preBötC produces normal respiratory activity (eupnea) as well as sighs under normoxic conditions, and it generates gasping under hypoxic conditions after a reconfiguration process. The reconfiguration leading to gasping generation involves changes of synaptic and intrinsic properties that can be mediated by several neuromodulators. Over the past years, it has been shown that endogenous continuous neuromodulation of the preBötC may involve the continuous action of amines and peptides on extrasynaptic receptors. I will summarize the findings supporting the role of endogenous continuous neuromodulation in the generation and regulation of different inspiratory rhythms, exploring the possibility that these neuromodulatory actions involve extrasynaptic receptors along with evidence of glial modulation of preBötC activity.

**Keywords:** endogenous neuromodulation, glia, network activity, pacemaker neurons, reconfiguration, extrasynaptic

## INTRODUCTION

Neural network activity relies on the interactions between intrinsic and synaptic properties (Ramirez et al., 2004; Marder and Bucher, 2007; Peña, 2009). Here, I will consider “synaptic properties” as those provided by fast neurotransmission among neurons and “neuromodulation” as the slower changes in cellular and synaptic properties mediated by metabotropic receptors (Katz, 1998). Neuromodulators regulate the activity of networks, allowing their adaptation to different demands or even conditioning their basal activity (Katz, 1998; Tryba et al., 2006; Peña, 2009). This issue has been studied in the inspiratory rhythm generator, the pre-Bötzinger complex (preBötC), which generates the inspiratory commands that control the diaphragm (Smith et al., 1991; Feldman and Del Negro, 2006; Schwarzacher et al., 2011). Breathing is eliminated by lesioning or inactivating the preBötC (Ramirez et al., 1998; Wenninger et al., 2004), whereas the isolated preBötC is still able to generate the inspiratory rhythms in a brainstem slice preparation (Smith et al., 1991; Lieske et al., 2000; Peña et al., 2008; Armstrong et al., 2010) or even in preBötC islands (Ramírez-Jarquín et al., 2012).

In slices, the preBötC generates three distinct activity patterns that correspond to distinct forms of breathing: normal respiratory activity (eupnea), sighs, and gasps (Lieske et al., 2000). Gasping is generated during hypoxia as a “last-resort” respiratory effort to autoresuscitate (Gozal et al., 2002; Fewell et al., 2007; Zavala-Tecuapetla et al., 2008). Interestingly, babies that die from SIDS have a reduction in gasping generation and inefficient autoresuscitation (Poets et al., 1999; Sridhar et al., 2003). The preBötC contains several types of neurons, including expiratory, inspiratory,

and postinspiratory neurons (Lieske et al., 2000) that interact through fast synaptic transmission (Greer et al., 1991; Funk et al., 1993; Shao and Feldman, 1997; Ren and Greer, 2006) to produce the inspiratory rhythms. Among the inspiratory preBötC neurons, a group of respiratory pacemaker neurons has been detected that plays a major role in rhythm generation (Thoby-Brisson and Ramirez, 2001; Peña et al., 2004; Del Negro et al., 2005; Peña and Aguileta, 2007). Characterization of these neurons revealed at least two types (Thoby-Brisson and Ramirez, 2001; Peña et al., 2004; Del Negro et al., 2005; Peña and Aguileta, 2007; Peña, 2008), one that generates bursts via a  $\text{Ca}^{2+}$ -activated cationic current ( $I_{\text{CAN}}$ ) and the other that relies on the persistent  $\text{Na}^{+}$  current ( $I_{\text{NaP}}$ ; Peña et al., 2004; Del Negro et al., 2005; Peña and Aguileta, 2007; Peña, 2008). Both types of pacemakers need to be inhibited to abolish rhythmogenesis under normoxia, both *in vitro* and *in vivo* (Peña et al., 2004; Peña and Ramirez, 2005; Tryba et al., 2006). In contrast, during hypoxic conditions, gasping generation critically relies on the activity of  $I_{\text{NaP}}$ -dependent (hypoxia-resistant) pacemaker neurons (Peña et al., 2004; Tryba et al., 2006; Peña and Aguileta, 2007). In addition to these mechanisms, the specific contribution of intrinsic and synaptic properties to rhythmogenesis depends on the neuromodulatory context. When applied exogenously, several neuromodulators modify the generation of the rhythmic activity by the preBötC (Doi and Ramirez, 2008). Moreover, several of these neuromodulators maintain a continuous endogenous modulation that, in some cases, is indispensable for rhythm generation (Peña and Ramirez, 2002, 2004; Tryba et al., 2006; Viemari et al., 2011; Ramírez-Jarquín et al., 2012). This continuous modulation, synonymous with “tonic neuromodulation,”

is maintained by continuous release of neuromodulators, mainly from tonic active neurons and glial cells (Hülsmann et al., 2000; Ptak et al., 2009).

### CONTINUOUS (“TONIC”) NEUROMODULATION OF THE preBötC

The actions of different neuromodulators on the inspiratory rhythm generator, including amines and peptides, were recently reviewed (Ballanyi, 2004; Peña and García, 2006; Doi and Ramirez, 2008; Peña, 2009). Therefore, I will focus on the evidence of endogenous continuous neuromodulations of the preBötC. In the CNS, several neuromodulators can be continuously released and act both at synaptic and extrasynaptic levels to regulate network function (Vizi et al., 2010). Extrasynaptic transmission was originally discovered for several monoamines that regulate the release of other neuromodulators and neurotransmitters despite the lack of synaptic contact between the two terminals (Vizi et al., 2010). In fact, the majority of monoaminergic and peptidergic neurons fail to make synaptic contacts and instead, they act on extrasynaptic receptors (Descarries and Mechawar, 2000; Vizi et al., 2010). Such neuromodulators are preferentially, but not exclusively, accumulated in large, dense-core vesicles, and they require a strong depolarization or high frequency stimulation to be released (Torrealba and Carrasco, 2004; De-Miguel and Trueta, 2005; Vizi et al., 2010). The fact that several neuromodulators, such as serotonin and adenosine, have been detected in the extracellular space of the preBötC by means of microdialysis (Richter et al., 1999), which detects neurotransmitters and neuromodulators that escaped the synaptic cleft (Peña and Tapia, 1999, 2000), suggests that they can reach extrasynaptic receptors and continuously modulate the preBötC. The extracellular concentration of these neuromodulators changes depending on the state of the network (i.e., hypoxia; Richter et al., 1999; Hehre et al., 2008) indicating that such continuous modulation adjusts the preBötC activity to fit particular demands. Next, I will present a catalog of neuromodulators that maintain a continuous neuromodulation of the preBötC, and discuss the possible involvement of extrasynaptic receptors or glial cells in this modulation. It is important to consider that respiratory rhythmogenesis is studied in a variety of experimental conditions ranging from behaving animals to preBötC islands (Ramírez-Jarquín et al., 2012). Thus, in most cases, the pharmacological manipulations could affect different respiratory circuits besides the preBötC (Zavala-Tecuapetla et al., 2008; Ramírez-Jarquín et al., 2012).

### ADENOSINE

Adenosine is an inhibitory neuromodulator of the preBötC (Schmidt et al., 1995; Herlenius and Lagercrantz, 1999; Wilken et al., 2000; Huxtable et al., 2009) that can be directly released from neurons and glia or that can be extracellularly produced by the degradation of released ATP (Martin et al., 2007; Cunha, 2008; Zwicker et al., 2011). Ambient adenosine can exert its effects by diffusing far away from the release sites (Cunha, 2008; Vizi et al., 2010). An adenosinergic continuous modulation of the preBötC of mice has been evidenced by blocking adenosine-receptors with the non-selective, adenosine-receptor antagonist aminophylline (Wilken et al., 2000), which increases the frequency and amplitude of inspiratory rhythm in slices. This effect is similar to blocking

the type 1 (A1) adenosine-receptor in rats with the specific antagonist DPCPX (Huxtable et al., 2009). These increases have also been observed in the brainstem-spinal cord preparation (also called the “*en bloc*”) of rats (Herlenius and Lagercrantz, 1999) and in cats *in vivo* (Schmidt et al., 1995), where levels of adenosine increase in hypoxia (Richter et al., 1999), contributing to the respiratory depression observed during this condition. In fact, blocking A1-receptors attenuates hypoxia-induced breathing in the *en bloc* of rats (Kawai et al., 1995). Thus, it has been suggested that adenosine antagonists can be useful for the treatment of several respiratory dysfunctions (Mathew, 2011).

### ATP

ATP excites the preBötC *in vitro* in rats (Huxtable et al., 2009; Zwicker et al., 2011) through the activation of P2Y-receptors (Lorier et al., 2007; Huxtable et al., 2009). Interestingly, blockade of endogenous activation of P2-receptors with suramin reduced inspiratory frequency in the slice preparation, while  $\text{Cu}^{2+}$ , an allosteric modulator of purinergic receptors, produced the opposite effect (Lorier et al., 2007, 2008). ATP is released during hypoxia, and blocking its tonic action on P2-receptors increases the hypoxia-induced slowing of the respiratory rhythm, suggesting that ATP is involved in maintaining respiration in hypoxia in rats (Gourine et al., 2005). Interestingly, the excitatory effect of exogenous ATP on the preBötC is precluded when glial cells are inhibited (Huxtable et al., 2009).

### ACETYLCHOLINE

Acetylcholine (ACh) is another neuromodulator that tonically regulates preBötC activity in rats and mice (Shao and Feldman, 2009). Application of the acetylcholinesterase inhibitor physostigmine increases the frequency of rhythmic respiratory activity in the slice preparation involving the type-3-muscarinic and  $\alpha 4\beta 2$ -nicotinic receptors in rats and mice, respectively (Shao and Feldman, 2005; Shao et al., 2008). Similarly, blockade of muscarinic-receptors with atropine reduces the amplitude and frequency of the respiratory rhythm in the *en bloc* from mice (Coddou et al., 2009). In the lamprey *en bloc*, physostigmine increases the respiratory frequency, while the nicotinic antagonists D-tubocurarine or bungarotoxin reduces it (Mutolo et al., 2011).

### NORADRENALINE

Pre-Bötzinger complex activity is modulated by endogenous noradrenaline released from the A5, A6, A1C1, and A2C2 nuclei in rats and mice (Hilaire et al., 2004; Viemari, 2008). This continuous modulation involves activation of  $\alpha$ -2-adrenoreceptors, since its blockade with yohimbine, piperoxane, or phentolamine decreases respiratory frequency in the *en bloc* in rats and mice (Errchidi et al., 1990; Zanella et al., 2006; Fujii and Arata, 2010) and abolishes gasping generation in slices from mice (Viemari et al., 2011). Accordingly, decreasing the extracellular noradrenaline concentration with pargyline, desipramine, or tyrosine increases the frequency of the rhythm, while methyltyrosine, an inhibitor of noradrenaline biosynthesis, increases the *en bloc* respiratory frequency in rats and mice (Errchidi et al., 1990; Zanella et al., 2006). There is some evidence of a continuous modulation of the preBötC by histamine and dopamine. Thus, the histamine-type-1-receptor antagonist, pyrilamine, reduces the *en bloc* respiratory

frequency and attenuates respiratory depression in hypoxia in mice (Dutschmann et al., 2003), while the dopamine-type-1-receptor antagonist SCH-23390 slows the respiratory rhythm of cats *in vivo* (Lalley, 2004, 2005).

### SEROTONIN

The preBötC is modulated by 5-hydroxytryptamine (5-HT), which produces an excitatory effect mediated by 5-HT<sub>2</sub>-receptors and an inhibitory effect mediated by 5-HT<sub>1</sub>-receptors (Schwarzacher et al., 2002). The main source of 5-HT is the raphe nuclei (Richerson, 2004), whose projections can or cannot make synaptic contacts with their targets throughout the brain (Kosofsky and Molliver, 1987). In the preBötC, increasing the extracellular concentration of 5-HT with 5-HT-uptake inhibitors leads to an increase of respiratory activity in the *en bloc* from rats (Di Pasquale et al., 1994). In contrast, blocking 5-HT-receptors with the non-specific antagonist methysergide abolishes rhythmogenesis in the *en bloc* and in slices from rats (Di Pasquale et al., 1994; Ptak et al., 2009). In these preparations, excitation of raphe neurons increases the frequency of the respiratory rhythm mediated by the activation of 5-HT<sub>2</sub>-receptors (Al-Zubaidy et al., 1996; Ptak et al., 2009). Accordingly, blocking either 5-HT<sub>2B</sub>-receptors (Günther et al., 2006), 5-HT<sub>2C</sub>-receptors (Ptak et al., 2009), or 5-HT<sub>2A</sub> receptors (Peña and Ramirez, 2002; Ptak et al., 2009) reduces the respiratory rhythm frequency and its regularity in slices from rats and mice. Such findings have been corroborated for 5-HT<sub>2A</sub>- and 5-HT<sub>2C</sub>-receptors *in situ* in rats (Ptak et al., 2009). Interestingly, low micromolar concentrations of 5-HT induce bursting activity in non-bursting preBötC neurons (Ptak et al., 2009), while blockade of 5-HT<sub>2A</sub> receptors abolishes the intrinsic bursting of the  $I_{NaP}$ -dependent (hypoxia-resistant) pacemaker neurons (Peña and Ramirez, 2002; Tryba et al., 2006). Consequently, blockade of 5-HT<sub>2A</sub> receptors inhibits gasping generation in slices from mice (Tryba et al., 2006) and *in situ* in rats (Bale and Solomon, 2010). These findings may have clinical relevance, since it has been hypothesized that a deficiency of the medullary 5-HT network is a potential cause of SIDS (Kinney et al., 2001).

### PEPTIDES

Several neuropeptides may exert a continuous regulation of the preBötC. Neuropeptides are typical non-synaptic transmitters, which are released extrasynaptically (Torrealba and Carrasco, 2004; Wotjak et al., 2008). Blocking the endogenous activation of the opioid-receptors with naloxone increases the respiratory output in cats (Lawson et al., 1979) and reduces hypoxia-induced respiratory depression in rats (Schlenker and Inamdar, 1995). In mice, blocking endogenous activation of somatostatin-receptors increases the respiratory rhythm frequency and reduces its regularity, both in slices and *in vivo* (Ramírez-Jarquín et al., 2012). Moreover, blockade of somatostatin-receptors, specifically subtype 2, prevents the reconfiguration of the preBötC during hypoxia *in vitro* and reduces gasping generation and autoresuscitation *in vivo* (Ramírez-Jarquín et al., 2012). In contrast, substance-P maintains an excitatory continuous modulation on the preBötC in rats and mice (Ptak et al., 2009; Doi and Ramirez, 2010). Blockade of the substance-P receptor (NK1) with SR 140333 or

spantide inhibits rhythmogenesis *in vitro* and *in situ* in mice and rats, respectively (Telgkamp et al., 2002; Ptak et al., 2009). Interestingly, in mice, inhibition of respiratory activity with NK1 antagonists has no significant respiratory effect when the levels of 5-HT or noradrenaline are increased by stimulating the raphe magnus or locus coeruleus, respectively (Doi and Ramirez, 2010), indicating that the action of substance-P might be influenced by the neuromodulatory state of the network (Doi and Ramirez, 2010).

### POSSIBLE REGULATION OF THE preBötC BY GABA AND GLUTAMATE ACTING ON EXTRASYNAPTIC RECEPTORS

Glutamatergic and GABAergic neurons were thought to release their transmitters exclusively at synapses, where they mediate the classical “fast synaptic transmission” (Vizi et al., 2010). However, it has been shown that ambient GABA and glutamate can also tonically activate high-affinity, extrasynaptic receptors, suggesting their spill-over from synaptic boutons, mediating a slower synaptic transmission (Semyanov et al., 2004; Farrant and Nusser, 2005; Aghajanian, 2009). Extrasynaptic GABA<sub>A</sub> inhibition can modulate the generation of hippocampal fast rhythms (Scanziani, 2000; Towers et al., 2004; Mann and Mody, 2010; Papatheodoropoulos and Koniaris, 2011), and it is likely that such modulation also occurs in the preBötC, where increasing the extracellular concentration of GABA, by inhibiting its uptake with nipecotic acid, decreases the respiratory frequency (Ren and Greer, 2006). The presence of delta-subunit-containing-GABA<sub>A</sub>-receptors, which are mainly extrasynaptic (Nusser et al., 1998; Adkins et al., 2001; Brown et al., 2002) suggests a tonic GABAergic control of the preBötC. For instance, the application of the GABA<sub>A</sub>-receptor agonist THIP, which preferentially activates extrasynaptic GABA<sub>A</sub>-receptors containing delta-subunits (Nusser et al., 1998; Adkins et al., 2001; Brown et al., 2002), hyperpolarizes respiratory neurons and reduces the frequency of the respiratory rhythm (Shao and Feldman, 1997). Neurosteroids, which also target delta-containing, extrasynaptic GABA<sub>A</sub>-receptors (Stell et al., 2003; Belelli and Lambert, 2005; Scimemi et al., 2006), modulate GABA<sub>A</sub>-receptor-mediated hyperpolarization of respiratory neurons and the inhibition of rhythmogenesis in slices (Ren and Greer, 2006).

Ambient glutamate can also activate extrasynaptic, NR2B-subunit-containing, NMDA-receptors and modulate neural network activity (Lambe and Aghajanian, 2006, 2007; Aghajanian, 2009). It is likely that extrasynaptic, NMDA-receptor-mediated excitation is also present in the preBötC, where inhibition of glutamate uptake with dihydrokainate increases rhythmogenesis (Greer et al., 1991; Funk et al., 1993). Dihydrokainate can also restore rhythmogenesis in substance-P-depleted slices, in which capsaicin abolishes rhythm generation (Morgado-Valle and Feldman, 2004). Similarly, releasing NMDA-receptors from their Mg<sup>2+</sup>-blockade restores rhythmogenesis in slices where the rhythm is abolished by AMPA-receptor blockade (Morgado-Valle and Feldman, 2007). This evidence supports the notion that a tone of extracellular glutamate can participate in rhythmogenesis. Furthermore, the presence of the NR2B-receptor has been extensively documented in the preBötC (Watanabe et al., 1994; Paarmann et al., 2000, 2005; Liu and Wong-Riley, 2010).

## GLIAL MODULATION OF THE preBötC

Glial cells are integral functional elements of neural networks, since it is argued that they can respond to and regulate neuronal activity (Araque and Navarrete, 2010). The respiratory network is not an exception (Gourine et al., 2010). Glial cells can sense preBötC activity, and a portion of them display a phase-locked rhythmic activity (Schnell et al., 2011). Moreover, glial cells are essential for rhythmogenesis, since both fluoroacetate, which selectively blocks the glial Krebs cycle, and methionine-sulfoximine, which blocks glutamine synthetase (Hülsmann et al., 2000; Young et al., 2005; Huxtable et al., 2010), inhibit rhythmic respiratory burst activity in slices. In these conditions, addition of isocitrate or glutamine restores the rhythmic network activity (Hülsmann et al., 2000). Accordingly, methionine-sulfoximine-treated pups displayed a reduced breathing frequency and a reduced responsiveness to hypercapnia (Young et al., 2005). Moreover, glial cells are required not only for maintaining rhythm generation but also for the response of the preBötC to neuromodulators or to metabolic demands (Gourine et al., 2010). For instance, fluoroacetate and methionine-sulfoximine reduce preBötC responsiveness to ATP (Huxtable et al., 2010), and preBötC glial cells can respond to preBötC neuromodulators including 5-HT and substance-P (Härtel et al., 2009).

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- I conclude that continuous neuromodulation exerts a powerful influence on the preBötC; to the extent that, in some cases, it is necessary for rhythm generation. Continuous neuromodulation tunes the excitability of the preBötC to respond to different demands and also determines the weight of specific neuronal types or specific synaptic interactions in the generation of network dynamics. This property could allow the preBötC to adopt an infinite number of conformations based on the same circuit (neural units and connections). Moreover, the evidence that one neuromodulation is determined by tonic control exerted by other neuromodulators, supports the notion that the intrinsic and synaptic properties of the preBötC are not fixed, but can change in a state-dependent manner. The levels of modulation in the preBötC would determine the availability of neural properties (intrinsic, synaptic, or both) that can participate in network dynamics or are susceptible to subsequent neuromodulation.

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